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Patentanmeldung Nr. Patent application No. Demande de brevet n°

99202048.7

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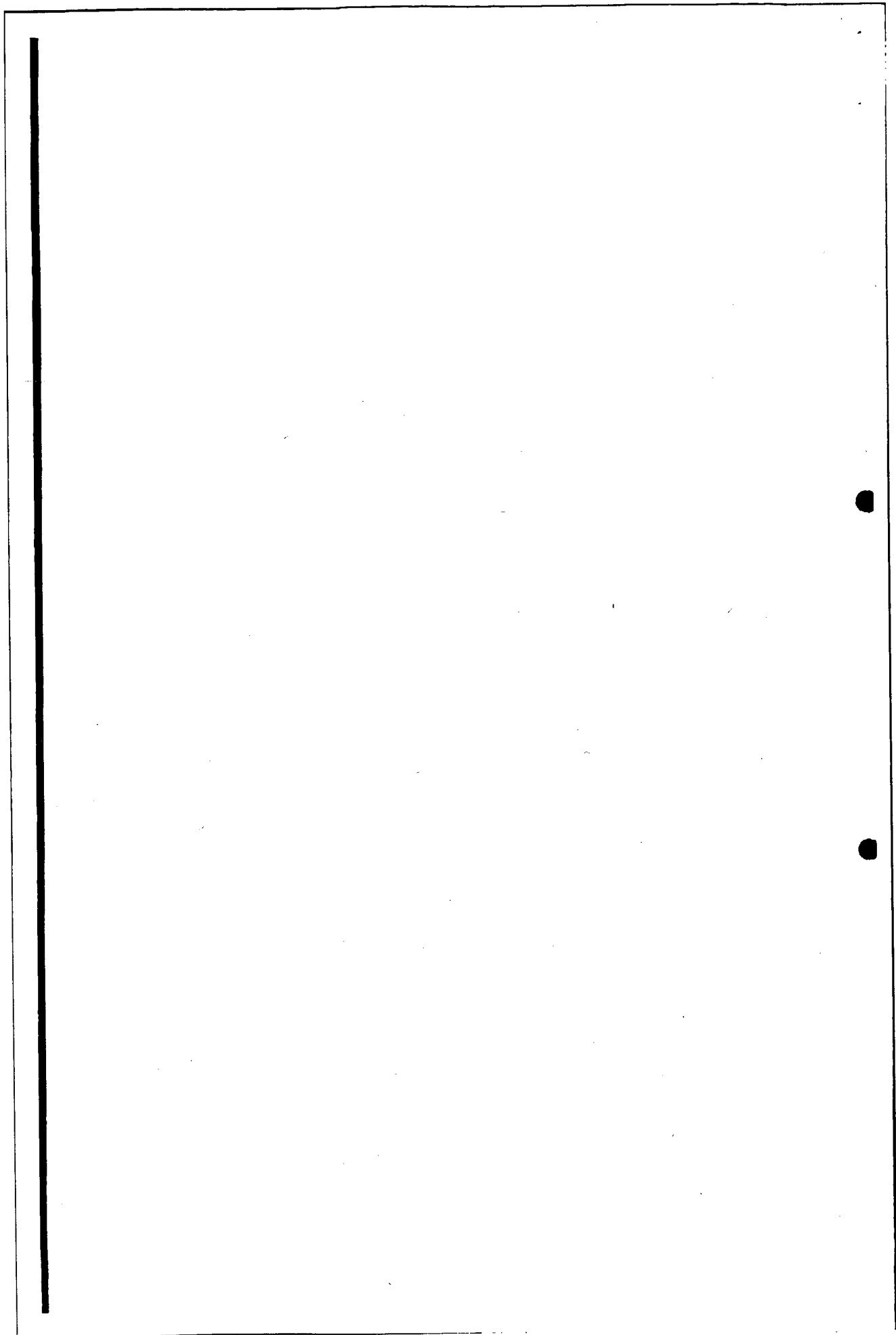
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Anmeldung Nr.
Application no
Demande n° 99202048.7

Anmeldetag
Date of filing
Date de dépôt 24/06/99

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Bezeichnung der Erfindung
Title of the invention
Titre de l'invention
Use of LPL S447X mutant in gene therapy

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat
State
Pays

Tag
Date
Date

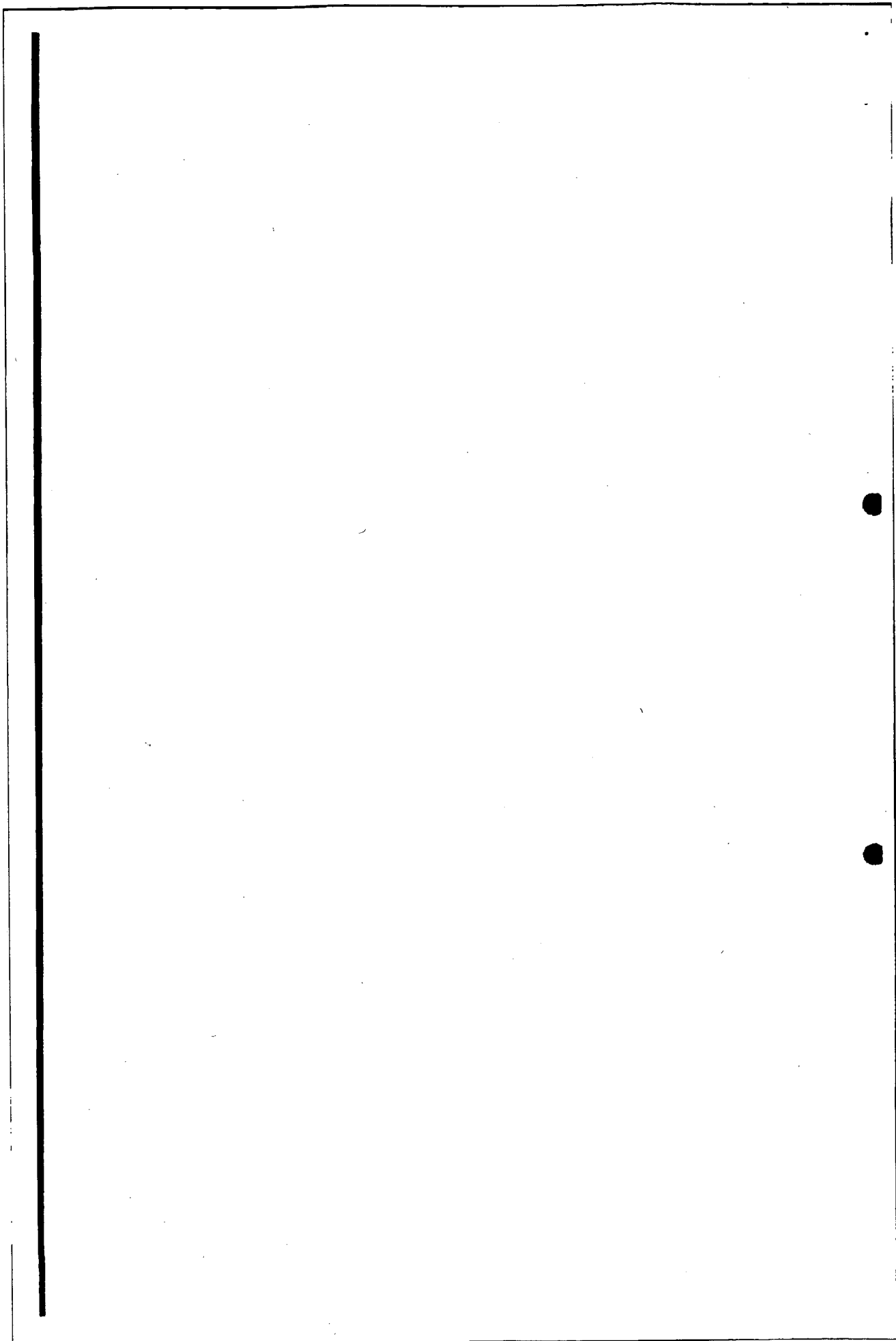
Aktenzeichen
File no
Numéro de dépôt

Internationale Patentklassifikation
International Patent classification
Classification internationale des brevets

/

Am Anmeldetag benannte Vertragsstaaten
Contracting states designated at date of filing AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE
Etats contractants désignés lors du dépôt

Bemerkungen
Remarks
Remarques



24. 06. 1999

USE OF LPL S447X MUTANT IN GENE THERAPY

(74)

The present invention relates to the use of the LPL S447X mutant in gene therapy.

5 The truncation variant S447X of wild type lipoprotein lipase (LPL) leads in the subject carrying the mutation to a higher LPL activity and lower triglycerides as well as higher HDL-cholesterol.

It was now found according to the present
10 invention that severe angina pectoris is associated with decreased lipoprotein lipase activity in plasma. Annex 1 attached hereto describes this finding in more detail.

In addition thereto it was found that the variant confers protection against coronary heart disease.
15 se. Annex 2 as attached hereto shows the details of this finding.

Furthermore, it was found that carriers of the LPL S447X mutation showed a lower blood pressure, both diastolic and systolic, than non-carriers. Annex 3 hereto
20 shows the results.

In view of the above the invention relates to the use of the LPL S447X mutant gene for the preparation of a gene therapeutic composition for the treatment of angina pectoris, for protection against cardiovascular
25 diseases and for lowering high blood pressure.

The LPL S447X mutant gene is incorporated in a suitable gene therapeutically acceptable vector, preferably an adeno-associated vector (AAV). Such a vector comprises for example a 5' inverted terminal repeat (ITR), a
30 promoter, preferably a CMV enhancer-promoter, with incorporated a muscle specific enhancer, an intron, a 3'-untranslated region (3'-UTR), a polyadenylation signal, preferably from SV40, and a 3'-ITR.

INTRODUCTION

Plasma triglyceride levels, as a marker for triglyceride-rich (TG) lipoproteins are now considered an established risk factor for coronary heart disease (CHD), independent of other lipoproteins.^{1,2}

Moreover, recent evidence suggests that elevated levels of these lipoproteins in the fasting or postprandial state promote the development of atherosclerotic plaques with lipid-rich cores that are particularly vulnerable to rupture.³

Since the new paradigm of CHD dictates that clinical prognosis is not mainly determined by the extent of a single stenosis, but more so by the biological composition of the plaque, TG-rich lipoproteins are increasingly considered as important contributors to this outcome.⁴

Therefore, both low-density lipoprotein (LDL) particles and TG-rich lipoproteins are now thought to contribute, in concert, to the cascade that causes atherosclerotic plaques to develop and ultimately predispose to CHD.

However, the initial event in atherogenesis constitutes reversible damage to the endothelium and it is noteworthy that dyslipidemia *per se* has direct deleterious effects on endothelial cells by reducing the bio-availability of nitric oxide.⁵ Even very modest elevations of LDL-C are associated with endothelial dysfunction which in its turn has now been demonstrated to lead to myocardial perfusion defects and ischemia.^{6,7}

In addition, it was recently reported that transient hypertriglyceridemia can also decrease endothelium-dependent vascular reactivity, suggesting a novel role for

TG-rich lipoproteins.⁸ These earlier observations are now supported by two recent studies that document impaired endothelium-dependent vasomotor responses in the coronary vasculature, again elicited by triglyceride-rich remnant lipoproteins.^{9,10}

Even a single high-fat meal, as a source of postprandial TG-rich lipoproteins, has now been shown to transiently impair endothelial function, induce red-cell aggregation, and promote disturbances in coagulation and fibrinolysis.^{11, 12}

Elevated TG-rich lipoprotein levels, therefore, do not only promote a more rapid progression of atherosclerosis but could also lead directly to myocardial ischemia, notably in subjects with the high TG-low HDL trait, frequently present in CAD patients, and an important marker for TG-rich lipoproteins.¹³

Lipoprotein lipase (LPL) is a crucial enzyme in the metabolism of these TG-rich lipoproteins. It is synthesized in parenchymal cells of adipose tissue and skeletal and cardiac muscle (CM), where it is transferred to binding sites at the vascular side of endothelial cells in nearby capillaries and epicardial vessels, the latter in case of the myocardium.¹⁴

We previously demonstrated that low levels of LPL activity, as encountered in patients with LPL deficiency, cause premature atherosclerosis and lead to increased progression of coronary atherosclerosis.^{15,16}

In contrast, patients with a genetically determined higher level of LPL activity exhibit lower triglycerides and higher HDL-cholesterol and are more frequently found among centenarians.^{17,18}

Since low levels of LPL-activity are strongly associated with the high TG-low HDL-C trait, we hypothesized that low LPL enzyme activity would not only predispose to premature CAD but also lead to endothelial dysfunction and ultimately to an increased frequency of myocardial ischemia in patients with CAD. We therefore initiated a test of this hypothesis by looking for an association between levels of LPL activity and measures of myocardial ischemia in a large cohort of white males, assessed by coronary angiography, who participated in a lipid lowering regression trial (REGRESS).¹⁹

MATERIALS AND METHODS

Patients

A total of 884 men taking part in the REGRESS study were eligible for this study. The REGRESS study, described in detail elsewhere was designed as a double blind, placebo-controlled, multi-centre study to assess the effect of pravastatin treatment on the progression and regression of coronary atherosclerosis.¹⁹ All patients were males of Caucasian descent, below 70 years of age, and had angiographically documented coronary artery disease (> 50% stenosis of one major vessel). Patients were specifically excluded who had unstable angina or who suffered a myocardial infarction within the preceding 6 months of the study. All patients had to have total cholesterol levels between 4 and 8 mmol/l and triglyceride levels below 4 mmol/l.

Lipid and lipoprotein analysis

All lipid laboratory tests were carried out at the Lipid Reference Laboratory, as published previously.¹⁹ Serum cholesterol, HDL cholesterol, and triglycerides were measured on fasting blood samples by standard techniques at all visits. LDL cholesterol was calculated according to the Friedewald formula.

The Lipid Reference Laboratory is an international member of the USA National Cholesterol Reference Method Laboratory Network, chaired by the Centers for Disease Control and Prevention, (Atlanta, GA, USA).

Quantitative Coronary Angiography (QCA)

The quantitative coronary angiography (QCA) procedures are described in detail elsewhere.¹⁹ Briefly, baseline coronary cinearteriography was performed 5 to 10 min. after oral administration of 5 to 10 mg isorbide dinitrate sublingually and analysed by QCA using the Cardiovascular Measurement System (CMS-MEDIS, Medical Imaging System). The coronary tree was divided in 13 segments according to the American Heart Association (AHA) classification, excluding the posterolateral branches. Minimum obstruction diameter (MOD), mean segment diameter (MSD), and percent diameter stenosis (% D-stenosis) were calculated for each qualifying segment. To calculate an average per patient, the MOD, MSD and % D-stenosis of all qualifying segments were added and divided by the number of contributing segments.

Holter Monitoring

Patients, physicians, and Holter technicians were blinded to the results of randomization throughout the study. The attending physicians were unaware of the results of the ambulatory (A) ECG. AECG monitoring was performed before randomization and after the intervention (PTCA or CABG). In the medical management group, the second recording was performed after 2 years. Not included in the AECG study were patients with initial ST-segment abnormalities,

for example, due to intraventricular conduction delay or right bundle-branch block. For the recording and analysis of transient myocardial ischemia, a three-channel Marquette system was used. During the time of the AECG, anti-ischemic medication was continued. Transient myocardial ischemia was defined as the presence of episodes showing ≥ 0.1 mV horizontal or downsloping ST-segment depression, 80 ms after the J-point, lasting for ≥ 60 seconds and separated by ≥ 60 seconds from the next ischemic episode. Ischemic burden was defined as the product of ischemic duration in minutes multiplied by ST-segment depression in millimeters. AECG recordings of poor technical quality were rejected, and recording periods in which the ST-segment altered due to a change in body position (during sleep) were not included in the study.

Statistical Analysis

LPL levels were studied in all REGRESS patients in whom a baseline LPL measurement was available.

The distribution of LPL activity was checked for its shape and transformed in order to stabilise and normalise it. The transformation was estimated using the Box-cox method. The association between LPL activity and baseline patient characteristics was assessed using ANOVA, univariate and multiple regression analyses. Similar methods were used to assess the relation between change in

LPL activity during the trial and these factors and change of LPL-activity and other parameters, such as change of diameter of the coronary vasculature.

In the assessment of these relations baseline levels were always used as adjustment to account for regression to the mean. Throughout a p-value of less than 0.05 was considered to indicate significance.

RESULTS

LPL activity measurements

Lipoprotein lipase (LPL) activities were available on baseline in 731 patients and at end of trial in 497 patients.

Square-root transformation normalised LPL distribution (Kolmogorov-Smirnov test: $p = 0.58$) and was used in all statistical inferences.

All 731 CAD patients were partitioned in three LPL activity groups; the first quartile (LPL-activity 13 - 77 mU/ml) contained 191 patients, the second and third quartile (LPL activity 77 - 132 mU/ml) contained 261 patients, and the fourth quartile (132 - 293 mU/ml) contained 179 patients. Mean baseline LPL activity was 107.0 ± 46 mU/ml.

Patient characteristics according to LPL activity

Distribution of CAD risk factors among LPL quartiles did not differ with regards to age, BMI, systolic and diastolic blood pressure, smoking, glucose, insulin, fibrinogen, or family history of premature CAD (data not shown).

Similar distributions were also found for pharmacological treatment in different LPL activity quartiles, i.e. for long-acting nitrates, beta blocking agents, calcium channel blockers and ACE inhibitors. In contrast, but as expected, CAD patients in the lowest LPL activity quartile displayed increased triglycerides (0.58 (0.43) vs. 0.35 (0.42) mmol/l (log transformed; $p < 0.0001$) and decreased HDL-

cholesterol (0.86 (0.26) vs. 1.02 (0.23) mmol/l; $p < 0.001$), indicative of the high TG-low HDL-C trait.

Coronary artery disease parameters according to LPL activity

Neither extent of CAD, nor baseline angiographical measurements differed between LPL activity quartiles (Table 1). However, New York Heart Association (NYHA) classification for angina pectoris was significantly different between LPL quartiles.

NYHA angina class could be scored in 875 out of 884 patients: 90 in Class I, 433 in II, 299 in III and 53 in class IV.

There was a highly significant difference in mean LPL level in patients going from NYHA class I to IV; means (SD) were 117 ± 47 , 114 ± 47 , 102 ± 43 and 83 ± 29 mU/ml, respectively ($p < 0.0001$) (Figure 1). After adjustment for angiographic and lipid parameters, risk factors and history of CAD, mean levels (SE) were: 117 ± 6.4 , 114 ± 2.8 , 104 ± 3.4 and 87 ± 7.9 mU/ml, respectively ($p = 0.002$). Patients in the highest NYHA class were, obviously, more often treated with anti-anginal medication and were subjected more often to cardiological intervention (CABG or PTCA). Even after adjusting for these factors, the differences in LPL activity between NYHA angina classes remained significant ($p = 0.013$). LPL activity levels were not, however, predictive of progression of disease in terms of change in MSD or MOD or cardiovascular events (data not shown).

Of patients in the lowest LPL activity quartile, 47% reported angina in class 3 or 4 and conversely only 29% in the highest LPL activity quartile had similar NYHA classification ($p < 0.001$). Angina class could be predicted by LPL activities in 67% of patients.

Subsequent analysis of 48 hour Holter monitoring confirmed subjective categorization and revealed a significant increase in both the number and duration of ischemic episodes, as well as an increased total ischemic burden (ST depression (mm) times ischemia (minutes)) in these low LPL-activity patients.

These differences between patients in the lowest versus the highest LPL activity quartile were highly significant (Table 1).

DISCUSSION

We could demonstrate in a large cohort of white males with established CAD, that LPL activity at baseline was not associated with dietary intake of fat, body weight, blood pressure, or extent of coronary atherosclerosis.

In contrast, CAD patients in the lowest quartile of LPL activity did exhibit higher TG and lower HDL-C-levels, and, in conjunction, suffered from twice as many episodes of silent ischemia as their counterparts in the highest quartile of LPL activity. In addition, and surprisingly, low LPL activity patients also reported severe angina pectoris twice as frequently as high LPL-activity patients.

Heparin releases LPL from its endothelial binding sites into the circulation and the amount of LPL in postheparin plasma is believed to reflect the amount of LPL that was exposed at the vascular endothelium in different tissues, including adipose tissue and skeletal and cardiac muscle.²⁰

Post-heparin LPL activity in our CAD patients, the largest cohort to-date in which these measurements were performed, was not associated with a number of environmental factors, but showed strong associations with lipids and measures of myocardial ischemia.

This indicates, in our opinion, that LPL activity should be considered a more innate individual property, not readily influenced by environmental factors, and

an important factor in the development and clinical sequelae of atherosclerotic vascular disease.

This is supported by the fact that heterozygotes for LPL deficiency, a condition by definition present from birth onwards, are more frequently found among patients with low LPL activity.²¹ Since the LPL enzyme represents the rate-limiting step for the removal of TG-rich lipoproteins from the circulation, the strong association in our cohort between low LPL activity and the high TG-low HDL trait can be easily understood and supports our earlier observations in LPL deficient heterozygotes.^{14,21,22}

The almost double frequency of angina and silent ischaemia in these low LPL activity patients, however, can be less readily explained.

We now know that nitric oxide (NO) contributes importantly to resting epicardial and coronary microvascular tone and that, even in the absence of angiographic evidence of atherosclerosis, exposure to TG-rich particles is associated with reduced bioavailability of NO from the coronary circulation.^{5,8-12, 23}

Our CAD patients had by definition, coronary atherosclerosis, and in that situation reduced availability of NO can give rise to myocardial perfusion defects, and ultimately lead to ischemia.

Nevertheless, the association between low LPL activity and myocardial ischemia cannot be fully explained by the increased presence of TG-rich lipoproteins in the circulation. The association between low LPL activity and angina is stronger than the association between low LPL activity and fasting levels of triglycerides.

However, humans are for the larger part of the day in the postprandial state and therefore assessment of fasting triglycerides may effectively underestimate the true strength of the association between LPL activity and TG levels.²⁴

Postprandial assessment of lipoprotein metabolism may provide a more physiologic perspective of dyslipidemia in these patients and may unmask significant hypertriglyceridemia, not evident in the fasting state, as was demonstrated for one particular mutation in the LPL gene.²⁵

Alternatively, one could hypothesize a direct relation between decreased LPL activity and vascular tone through modulation of the synthesis or degradation of NO. It is well established that endothelium-dependent vascular relaxation is abnormal in the setting of atherosclerosis, since less endothelial cells express nitric oxide synthase (NOS), the key enzyme in basal endothelial cell NO production.²⁶

LPL induces increased nitric oxide synthetase (NOS) mRNA expression and NO production in macrophages and we hypothesize that LPL has a similar function in the arginine metabolism and NO production of endothelial cells.²⁷

Our patients have angiographically proven atherosclerosis and therefore deficient NO production in the coronary vasculature. Those particular patients with low LPL activity would, in our hypothesis be at even greater disadvantage since both indirectly through increased levels of both fasting and postprandial TG-rich lipoproteins and directly through modulation of NO synthase, their capacity to synthesize NO is further diminished and myocardial perfusion is jeopardized at more modest levels of physical exertion.

Since experimental ischemia actually decreases LPL activity in the myocardial circulation, a situation could arise where a pre-existent low LPL activity at the endothelial surface is even further decreased by ischaemia which in turn elicits lower NO production and deteriorates into a vicious circle.

Table 1. Coronary artery disease parameters according to LPL activity

Patients (n)	LPL < 77 191	LPL >77 < 132 361	LPL > 132 179	p-value
<i>Extent of CAD</i>				
1 vessel	69 (43%)	125 (39%)	76 (47%)	0.32
2 vessel	49 (30%)	119 (37%)	49 (30%)	0.32
3 vessel	44 (27%)	73 (24%)	36 (22%)	0.32
<i>Angina Class</i>				
I	22 (12%)	32 (9%)	25 (14%)	< 0.001
II	79 (42%)	180 (50%)	104 (68%)	< 0.001
III	66 (35%)	123 (34%)	48 (27%)	< 0.001
IV	22 (12%)	23 (6%)	2 (2%)	< 0.001
<i>Angiography</i>				
MSD (mm)	2.75 (0.39)	2.75 (0.37)	2.72 (0.39)	0.69
MOD (mm)	1.76 (0.33)	1.77 (0.37)	1.75 (0.36)	0.89
<i>Ischemia</i>				
Duration (min)	16.2 (43.4)	7.2 (21.5)	8.3 (22.2)	0.013
Episodes (n)	2.6 (6.5)	1.3 (3.6)	1.3 (2.8)	0.012
Burden (min. n)	36.5 (104.1)	12.3 (37.6)	14.8 (38.8)	0.001

LPL= lipoprotein lipase; CAD= coronary artery disease; MSD= mean segment diameter; MOD= mean obstruction diameter.

LITERATURE

1. Hokanson JE, Austin MA. Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population-based prospective studies. *J Cardiovasc Risk* 1996; 3: 213-219.
2. Gaziano JM, Hennekens CH, O'Donnell CJ, Breslow JL, Buring JE. Fasting triglycerides, high-density lipoprotein, and risk of myocardial infarction. *Circulation* 1997; 96: 2520-2525.
3. Gronholdt MLM, Nordestgaard BG, Wiebe BM, Wilhjelm JE, Sillesen H. Echo-lucency of computerized ultrasound images of carotid atherosclerotic plaques are associated with increased levels of triglyceride-rich lipoproteins as well as increased lipid content. *Circulation* 1998; 97: 34-40.
4. Davies MJ. Stability and instability: two faces of coronary atherosclerosis. The Paul Dudley White lecture. *Circulation* 1996; 94: 2013-2020.
5. Quyyumi AA, Dakak N, Andrews NP, Husain S, Arora S, Gilligan DM, Panza JA, Cannon III RO. Nitric Oxide activity in the human coronary circulation. *J Clin Invest* 1995; 95: 1747-1755.
6. Steinberg HO, Bayazeed B, Hook G, Johnson A, Cronin J, Baron AD. Endothelial dysfunction is associated with cholesterol levels in the high normal range in humans. *Circulation* 1997; 96: 3287-3293.

7. Hasdai D, Gibbons RJ, Holmes Jr DR, Higano ST, Lerman A. Coronary endothelial dysfunction in humans is associated with myocardial perfusion defects. *Circulation* 1997; 96: 3390-3395.
8. Lundman P, Eriksson M, Schenck-Gustafsson K, Karpe F, Tornvall P. Transient triglyceridemia decreases vascular reactivity in young, healthy men without risk factors for coronary heart disease. *Circulation* 1997; 96: 3266-3268.
9. Kugiyama K, Doi H, Ohgushi M, Motoyama T. Remnant lipoproteins impair endothelium-dependent vasomotor functions in human coronary arteries. 70th Scientific Sessions of the AHA; 2207.
10. Inoue T, Riichiro M, Kazuhiro H, Issa Y, Shigenori M. Serum levels of remnant like particles - Cholesterol affects the endothelium dependent coronary vascular response in humans. 70th Scientific Sessions of the AHA; 2208.
11. Vogel RA, Corretti MC, Plotnick GD. Effect of a single high fat meal on endothelial function in healthy subjects. *Am J Cardiol* 1997; 79 350-354.
12. Baliga R, Rampling MW, Kooner JS. High fat meal induces changes in blood rheology in patients with coronary artery disease. 70th Scientific Sessions of the AHA; 2206.
13. Hodis HN, Mock WJ. Triglyceride-rich lipoproteins and progression of coronary artery disease. *Curr Opin Lipid* 1995; 6: 209-214.
14. Brunzell JD. Familial lipoprotein lipase deficiency and other causes of the chylomicronemia syndrome. In: Scriver CR, Beaudet AL, Sly WS, Valle D,

- eds. The Metabolic and Molecular Bases of Inherited Disease. New York, NY: McGraw-Hill, Inc: 1995; 1913-1932.
15. Benlian P, de Gennes JL, Foubert L, Zhang H, Gagné SE, Hayden MR. Premature atherosclerosis in patients with familial chylomicronemia caused by mutations in the lipoprotein lipase gene. *New Engl J Med* 1996; 335: 848-854.
16. Jukema JW, van Boven AJ, Groenemeijer BE, Zwinderman AH, Reiber JHC, Bruschke AVG, Henneman JA, Molhoek GP, Bruin T, Jansen H, Gagné SE, Hayden MR, Kastelein JJP. The Asp₉Asn mutation in the lipoprotein lipase gene is associated with increased progression of coronary atherosclerosis. *Circulation* 1996; 94: 1913-1918.
17. Groenemeijer BE, Hallman MD, Reymer PWA, Gagne SE, Kuivenhoven JA, Bruin T, Jansen H, Lie KI, Bruschke AVG, Boerwinkle E, Hayden MR, Kastelein JJP. Genetic variant showing a positive interaction with β -blocking agents with a beneficial influence on lipoprotein lipase activity, HDL cholesterol, and triglyceride levels in coronary artery disease patients. *Circulation* 1997; 95: 2628-2635.
18. Faure Delanef F, Zuoali H, Cohen D, Béreziat G, Benlian P. Prevalence of common lipoprotein lipase (LPL) gene mutation Ser447stop is increased while prevalence of Asn2911Ser mutation is decreased in centenarians. *Atherosclerosis* 1997; 134: 1.P.26.
19. Jukema JW, Bruschke AVG, van Boven AJ, Reiber JHC, Bal ET, Zwinderman AH, Jansen H, Boerma GJM, van Rappart FM, Lie KI. Effects of lipid lowering by pravastatin on progression and regression of coronary artery

- disease in symptomatic men with normal to moderately elevated serum cholesterol levels; the Regression Growth Evaluation Statin Study (REGRESS). *Circulation* 1995; 91: 2528-2540.
20. Olivecrona G, Hultin M, Savonen R, Skottova N, Lookene A, Tugrul Y, Olivecrona T. Transport of lipoprotein lipase in plasma and lipoprotein metabolism. In: *Atherosclerosis X*. P.P. Woodford, J. Davignon and A. Sniderman, eds. pp. 250-253.
21. Reymer PWA, Gagné E, Groenemeijer BE, Zhang H, Forsyth IJ, Jansen H, Seidell JC, Kromhout D, Lie KI, Kastelein JJP, Hayden MR. A lipoprotein lipase mutation (Asn291Ser) is associated with reduced HDL cholesterol levels in premature atherosclerosis. *Nature Genetics* 1995; 10: 28-34.
22. Kastelein JJP, Groenemeijer BE, Henderson H, Reymer PWA, Gagné SE, Seidell JC, Kromhout D, Jukema JW, Bruschke AVG, Boerwinkle E, Hallman MD. The -93/Asn9 haplotype of the lipoprotein gene increases risk for coronary artery disease. *Clinical Genetics*, in press.
23. Stroes E, de Bruin TWA, de Valk H, Erkelens WE, Banga JD, van Rijn H, Koomans H, Rabelink T. No activity in familial combined hyperlipidemia: potential role of cholesterol remnants. *Cardiovasc. Res.* 1997; 36: 445-452.
24. Cohen JC, Noakes TD and Spinnler Benade AJ. Postprandial lipemia and chylomicron clearance in athletes and in sedentary men. *Am. J. Clin. Nutr.* 1998; 49: 443-447.
25. Pimstone SN, Clee SM, Gagné SE, Miao L, Zhang H, Stein EA and Hayden MR. A frequently occurring mutation in the lipoprotein lipase gene

(Asn291Ser) results in altered postprandial chylomicron triglyceride and retinyl palmitate response in normolipidemic carriers. *J. Lipid Res.* 1996; 37: 1675-1684.

26. Wilcox JN, Subramanian RR, Sundell CL, Tracey WR, Pollock JS, Harrison DG, Marsden PA. Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels. *Arterioscler. Thromb. Vasc. Biol.* 1997; 17: 2479-2488.

27. Reinier G, Lambert A. Lipoprotein synergizes with interferon gamma to induce macrophage nitric oxide synthetase mRNA expression and nitric oxide production. *Arterioscler Thromb Vasc Biol* 1995; 15: 392-399.

Annex 2

ABSTRACT

Genetic variation at the lipoprotein lipase (LPL) locus has been shown to influence plasma lipids and to modulate risk of coronary heart disease (CHD). Recently, we found that the most frequent variant at this locus, involving a C-terminal truncation of two amino acids (Scr447X), was associated with both higher LPL activity and HDL-cholesterol in patients with CHD. However, the impact of this S447X variant on lipids and CHD in the general population was hitherto unknown. We therefore analyzed a total of 1114 men and 1144 women randomly ascertained from the Framingham Offspring Study (FOS) for the presence of this LPL variant. Carrier frequency of the S447X allele was 17%, and in men carrier status was associated with higher total cholesterol (TC) ($\Delta=6.2$ mg/dl, $p=0.03$), higher HDL-C ($\Delta=2.3$ mg/dl, $p=0.01$) and lower triglyceride (TG) levels ($\Delta=-19.4$ mg/dl, $p=0.02$). Moreover, in men the S447X allele conferred significant protection against CHD (odds ratio: 0.43; $p=0.04$). These effects on lipids and CHD were not seen in women. Our study represents the first report on the impact of this mutation on CHD in men from the general population, and we conclude, therefore, that the S447X variant may confer significant protection against high TG levels, low HDL-C and premature CHD in these subjects.

INTRODUCTION

Evidence from twin studies indicates that genetic factors play a major role in susceptibility to atherosclerosis (1). Genetic variation in lipoprotein levels primarily manifests as elevated LDL-C and decreased HDL-C, both associated with an increased risk for atherosclerotic

Annex 2

vascular disease (2,3). In addition, it is now also accepted that increased levels of plasma triglycerides (TG) constitute a risk factor for CHD (4). Lipoprotein lipase (LPL) is a pivotal enzyme in lipoprotein metabolism, is a recognized determinant of both plasma TG and HDL-C levels, and is therefore represents in important candidate for atherogenesis (5).

We, and others, have reported that three common variants in the gene for LPL, N291S, D9N and S447X (premature truncation at codon 447) are associated with altered levels of both TG and HDL-C (6-11). We have specifically shown that the N291S variant leads to a decrease in HDL levels in both CHD patients (6) while the D9N variant is associated with higher TG levels and leads to more rapid progression of coronary atherosclerosis (9).

In contrast, we have shown that the Ser447X variant, occurring at a high frequency in Caucasians, is associated with lower triglyceride and higher HDL-C levels in both normolipidemic men and CHD patients (10,11).

It is unknown whether this latter variant can influence lipids or CHD in the general population. We therefore sought to assess the relation of the S447X allele to lipid levels and to CHD in a representative sample from the general population, the Framingham Offspring Cohort.

METHODS

Population, Lipid and DNA Analyses

Details of the study design of the FOS have been published previously (12). All laboratory specimens were obtained during FOS examination cycle four, between April 1987 and September 1991. CHD was defined as angina pectoris, myocardial infarction, or coronary insufficiency, as published previously (12).

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Blood was drawn after a 12 hour fast for the determination of plasma glucose and lipids according to a modified Lipid Research Clinic Protocol (13). Human DNA was extracted from leukocytes by standard procedures.

Among 4019 participants at FOS cycle four, DNA was obtained on 3415 subjects, and 2416 were randomly sampled for LPL genotyping; sampled and non-sampled subjects did not differ materially. LPL genotyping was performed as previously described (10). In all, 158 subjects were excluded – incomplete genotypes (n=21), use of cholesterol-lowering medication (n=93), incomplete clinical data (n=44) – leaving 2,258 subjects for analysis.

Statistics

Linear regression models that included age, BMI, alcohol, fasting glucose, diabetic status, systolic and diastolic blood pressure, anti-hypertensive therapy, and smoking (plus menopausal status and estrogen therapy for women) were used for analysis of lipid values (14). Age splines were used to accommodate nonlinear age patterns (different slopes were fitted for <45 years of age, 45 to 59, and 60 or older). for analysis of lipid data, linear splines were fitted with knots at 44 and 59 years of age. What this means is that the regression of lipid values versus age was allowed to have one slope up to 44 years of age, a second slope between 44 and 59 years of age, and a third slope over 59 years of age. These age cut points were chosen because they are the 25th and 75th percentiles of the age distribution in the sample.

For analysis of CHD status, a single slope (of log odds ratio) versus age was used, because few subjects with CHD were under 60 years of age, such that using splines gave unstable/unreliable estimates.

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Further analyses used mixed-model linear regressions to accommodate correlations within sibships. Logistic regression was employed for associations between the LPL variant and CHD (15); due to paucity of multi-case sibships, we did not adjust the CHD analyses for correlated data within sibships.

To test for interactions of LPL genotype with age and BMI, secondary analyses were conducted using linear or logistic regression models, augmented by interaction variables. Statistical analyses were performed with SAS procedures REG, MIXED and LOGISTIC (16). A two-sided p-value of $p < 0.05$ was considered significant.

RESULTS

Frequency of the S447X variant

Genotyping yielded 1879 non-carriers, and 358 heterozygous and 21 homozygous carriers for the LPL S447X truncation variant. The S447X LPL variant was present in 16.1% of men, and 17.5% of women. Allele frequencies were in accordance with Hardy-Weinberg equilibrium (17). In subsequent analyses, heterozygotes and homozygotes were pooled.

Demographic Characteristics

The 179 male carriers were fully comparable to 935 non-carriers with regards to age, body-mass index, systolic and diastolic blood pressure, glucose levels, alcohol intake, and percentage of persons with diabetes, hypertension or smokers. Similarly, all baseline characteristics were comparable for the 200 female carriers versus the 944 non-carriers, except for body-mass index (lower in carriers; 25.2 kg/m^2 ($p=0.002$)). Mean (SD) TC levels were 203.5(36.3)mg/dl and 203.1(38.9)mg/dl, mean (SD) LDL levels were 133.4(32.2)mg/dl and

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125.3(34.7)mg/dl, mean (SD) HDL levels were 43.8(11.6)mg/dl and 56.3(15.3)mg/dl and mean (SD) TG levels were 136.7(102.4)mg/dl and 107.9(85.1)mg/dl for men and women, respectively.

Effect of the S447X variant on plasma lipids

Male carriers of the S447X allele (179 men) showed higher TC levels (+6.2mg/dl; $p=0.03$), LDL-C (+6.5mg/dl; $p=0.01$), HDL-C (+2.3mg/dl; $p=0.01$) and lower triglyceride levels (-19.4mg/dl; $p=0.01$) (Table 1). Percentage mean differences in male carriers were TC +3.5%, LDL-C +5.6%, HDL-C +5.3% and TG -13.8% when adjusted for age, BMI, alcohol and smoking status. Differences in HDL values between male carriers and non-carriers were also evident for HDL subclass 3; 40.1(0.61)mg/dl vs. 38.3(0.27)mg/dl; $p<0.01$. We also tested for interactions of the S447X genotype with smoking, age, and body-mass index (grouped into 1st, 2nd and 3rd, and 4th quartile), with regards to TC, HDL-C and TG. Among 18 interaction tests, no p-values below 0.01 were found.

Corresponding analyses in women showed no association with this LPL variant with regard to lipids in the group as a whole nor when stratified based on their menopausal status.

All analyses were repeated with mixed-model regressions to accommodate correlated data within families but no material alteration in results was obtained.

The S447X variant and risk of CHD

CHD was present in 84 men (8%) and 36 women (3%). After adjusting for age, BMI, smoking, alcohol (and menopausal status and estrogen therapy in women), the S447X allele is associated with significant protection against CHD in men but not in women, in the whole group (Table 2). Analysis in postmenopausal women only of the relation S447X carrier status with

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presence of CHD yielded an odds ratio of 1.00 (95% CI 0.36-2.74; $p=0.99$) confirming our data for the whole group.

DISCUSSION

A predisposition to altered lipoprotein metabolism and premature cardiovascular disease is likely to involve the interaction of multiple genes and environmental factors. One such factor is lipoprotein lipase (LPL) which has been shown to influence plasma lipids and to modulate risk of coronary heart disease (CHD) (6-9).

However, the frequency and phenotypic effects of the most common genetic variant of LPL, a premature truncation variant, S447X, has been studied in CAD patients but not in the general population (10,11). Specifically, we report here that approximately 17% of Framingham inhabitants are carriers of this truncated LPL. Similar frequencies for the S447X variant were found in three previous studies in population sin Europe (10,11,18). It therefore seems likely that our findings can be extended to North Americans of European descent. In addition, we show that this LPL mutant is associated with higher HDL-C and lower TG in men. Most importantly though, we could demonstrate that this common variant protects against the development of CHD in men.

LPL is a crucial enzyme in the generation of both HDL and LDL particles, as is demonstrated by the near absence of these lipoproteins in congenital LPL deficiency. Increased activity of LPL, therefore, could theoretically lead to increased levels of both lipoproteins. However, since both the ratio of TC to HDL-C (4.9 vs. 5.0) and the ratio of TG to HDL-C (3.1 vs. 3.8) are less in S447X carriers than in non-carriers, CHD-risk is decreased, despite a slight

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increase in plasma LDL-cholesterol. Moreover, the combination of decreased TG and increased HDL in S447X carriers will likely lead to an LDL subfraction profile of lower density, which is in turn associated with decreased CHD risk.

Recently, it was also reported that the S447X variant may result in increased production of LPL protein and higher lipolytic activity (11,19). Increased lipolysis results in enhanced clearance of atherogenic remnant particles and reduction of these particles could provide another possible mechanism for the effect of this variant on lipids and CHD.

We have, however, not excluded the fact that the S447 may have other effects on atherogenesis independent of its effects on lipids. This includes potential direct effects on the vessel wall as well as an influence on the uptake of lipoproteins by different receptors.

No significant associations between this LPL variant and plasma lipids or CHD could be demonstrated in women. The exact reasons for this are uncertain, but a lack of statistical power due to insufficient numbers to assess CHD incidence seems likely, while in contrast, gender specificity could offer an explanation for the observed discrepancy in lipid levels, as was recently demonstrated for another LPL mutation (7).

Results in postmenopausal female carriers vs. non-carriers with regards to lipids, lipoproteins and odds for CHD were similar as in the entire group of women.

A limitation of our current analysis is the fact that it constitutes a cause-control analysis of prevalent CHD, introducing potential bias when case fatality rates in mutation carriers are suspected to be higher than those in non-carriers. This seems, however, highly unlikely. Since 1 out of 5 persons carry either of the three common LPL variants, a single multiplex DNA-

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diagnostic test would be the best and cheapest method to determine carriership for any of these three in a cost-effective manner.

In conclusion, here we describe a common polymorphism in the LPL gene which modulates lipid levels in the general population. In addition, we report for the first time that a common DNA change seen in 1 in 6 Caucasian men in North America is associated with protection against premature atherosclerosis. Such results encourage further examination of the role of LPL in CHD and the potential benefits of assessment for this mutation in the general population.

REFERENCES

1. Marenberg ME, Risch N, Berkman LF, Floderus B, de Faire U. Genetic susceptibility to death from coronary heart disease in a study of twins. *New Engl J Med* 1994; 330: 1041-1046.
2. Wilson PW, Garrison RJ, Castelli WP, Feinleib M, McNamara PM, Kannel WB. Prevalence of coronary heart disease in the Framingham Offspring Study: role of lipoprotein cholesterol. *Am J Cardiol* 1980; 46: 649-654.

Annex 2

3. Assmann G, Schulte H. Relation of high-density lipoprotein cholesterol and triglycerides to incidence of atherosclerotic coronary artery disease (the PROCAM experience). Prospective Cardiovascular Münster Study. *Am J Cardiol* 1992; 70: 733-737.
4. Hokanson JE, Austin MA. Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population-based prospective studies. *J Cardiovasc Risk* 1996; 3: 213-219.
5. Taskinen MR, Nikkila EA. High density lipoprotein subfractions in relation to lipoprotein lipase activity of tissues in man - evidence for reciprocal regulation of HDL2 and HDL3 levels by lipoprotein lipase. *Clin Chim Acta* 1981; 112: 325-332.
6. Reymers PWA, Gagne E, Groenemeyer BE, et al. A lipoprotein lipase mutation (Asn291Ser) is associated with reduced HDL cholesterol levels in premature atherosclerosis. *Nat Genet* 1995; 10: 28-34.
7. Wittrup HH, Tybjaerghansen A, Abildgaard S, Steffensen R, Schnohr P, Nordestgaard BG. A common substitution (Asn291Ser) in lipoprotein lipase is associated with increased risk of ischemic heart disease. *J Clin Invest* 1997; 99: 1606-1613.
8. Mailly F, Tugrul Y, Reymers PWA, et al. A common variant in the gene for lipoprotein lipase (Asp9-Asn). Functional implications and prevalence in normal and hyperlipidemic subjects. *Arterioscler Thromb Vasc Biol* 1995; 15: 468-478.
9. Jukema JW, van Booven AJ, Groenemeijer B, et al. The Asp9Asn mutation in the lipoprotein lipase gene is associated with increased progression of coronary atherosclerosis. *Circulation* 1996; 94: 1913-1918.

Annex 2

10. Kuivenhoven JA, Groenemeyer BE, Boer JM, et al. Ser447stop mutation in lipoprotein lipase is associated with elevated HDL cholesterol levels in normolipidemic males. *Arterioscler Thromb Vasc Biol* 1997; 17: 595-599.
11. Groenemeyer BE, Hallman MD, Reymers PWA, et al. Genetic variant showing positive interaction with beta blocking agents with a beneficial influence on lipoprotein lipase activity, HDL cholesterol, and triglyceride levels in coronary artery disease patients; The Ser⁴⁴⁷Stop substitution in the lipoprotein lipase gene. *Circulation* 1997; 95: 2628-2635.
12. Feinleib M, Kannel WB, Garrison RJ, McNamara PM, Castelli WP. The Framingham Offspring Study. Design and preliminary data. *Prevent Med* 1975; 4: 518-525.
13. Lipids Research Clinics Program. Manual of Laboratory Operations. Bethesda, MD: National Institute of Health; NIH Publication, 1974: 75-686.
14. Kleinbaum DG, Kupper LL, Muller KE. Applied Regression Analysis and Other Multivariable Methods. 2nd ed. Boston, MA: PWS-Kent Publishing Company, 1988: 102-340.
15. Hosmer DW, Lemeshow S. Applied Logistic Regression. New York: John Wiley and Sons, 1989: 1-175.
16. SAS Institute Inc., SAS/STAT Software: Changes and Enhancements through Release 6.11. Cary, NC: SAS Institute, Inc., 1996.
17. Khoury MJ, Beary TH, Cohen BH. Fundamental of genetic epidemiology. Oxford University Press, Oxford 1993: 49-54.

Annex 2

18. Stocks J, Thom JA, Galton DJ. Lipoprotein lipase genotypes for a common premature termination codon mutation detected by PCR mediated site-directed mutagenesis and restriction digestion. *J Lipid Res* 1992; 33: 853-857.
19. Zhang, H, Henderson H, Gagné SE, Miao L, Liu G, Hayden MR. Common sequence variants of lipoprotein lipase: Standardized studies of in vitro expression and catalytic function. *Biochim Biophys Acta*. 1996; 1302: 159-166.

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Table 1: Effect of the S447X allele on plasma lipids by gender

	S447X Carriers	Non-carriers	
	Mean \pm se	Mean \pm se	p value
Men n=1114	n=179	n=935	
Total-C	208.6 \pm 2.6	202.5 \pm 1.1	0.031
LDL-C	138.7 \pm 2.4	132.3 \pm 1.0	0.013
HDL-C	45.7 \pm 0.8	43.4 \pm 0.3	0.008
Triglycerides	120.3 \pm 7.2	139.8 \pm 3.2	0.014
Triglyceride/HDL-C	3.1 \pm 0.3	3.8 \pm 0.1	0.019
Women n=1144	n=200	n=944	
Total-C	202.0 \pm 2.5	203.3 \pm 1.1	0.65
LDL-C	123.4 \pm 2.3	125.6 \pm 1.0	0.37
HDL-C	56.6 \pm 0.9	56.2 \pm 1.0	0.37
Triglycerides	112.4 \pm 5.4	106.9 \pm 2.5	0.36
Triglyceride/HDL-C	2.6 \pm 0.2	2.3 \pm 0.1	0.14

Adjusted for age splines, BMI, alcohol, smoking, glucose, diabetes, blood pressure levels and hypertension treatment in men, plus menopause and hormonal replacement therapy in women.

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Table 2: Odds ratio(OR) and preventive fraction (PF) of S447X carriers for CHD

		CHD disease		Odds Ratio (95% CI)	PF (%)	p value
		Non-Carriers	Carriers			
men	(n=1114)	77/935(8.2%)	7/179(3.9%)	0.43 (0.19-0.98)	9.6	0.044
women	(n=1144)	30/944(3.2%)	6/200(3.0%)	0.95 (0.38-2.41)	0.8	0.92

PF = preventive fraction was calculated as $100 \times P(OR-1)$ where P=proportion of carriers. Models included age, BMI, alcohol intake, smoking status plus menopausal status and hormone replacement therapy in women.

LPL S447X variant in familial hypercholesterolemia: lipids, vascular disease and blood pressure**Total cohort of 407 heterozygous FH patients after exclusion of:**

- all those on meds that might affect lipids (lipid lowering, diuretics, β -blockers, HRT, steroids, anti-epileptics) → have pre-medication lipid levels
- those with diabetes or impaired glucose tolerance
- those who were homozygous for the apoE2 allele
- carriers of the LPL 207 or 188 mutations (in French Canadians)
- carriers of the LPL D9N or N291S variants
- those who were pregnant
- those with kidney or liver disease
- those <18 yrs of age

Baseline demographics- Table 1.

- no difference in mean age, BMI, glucose, blood pressure, or male to female ratio between carrier and non-carrier groups

Lipid results- Table 2.

- carriers have significantly lower TG (and log-transformed TG)
- also significantly lower TG/HDL
- no difference in HDL cholesterol

Vascular disease

- diagnosed as CHD, PVD and/or CVA (MI, CABG, PTCA, angina treated with medication, CHD on angiogram, stroke, TIA, claudication and surgery on carotid or abdominal arteries treated due to atherosclerosis; does not include bruits only, aneurysms, ultrasound results only or other ambiguous diagnoses)

- looking in the above cohort, there was no difference in vascular disease between the groups (table 2)
- but with the exclusion of those on meds, several vascular disease cases were excluded; repeated the analysis with these individuals included again
- 2 variations on the stats, still no significant difference (Table 3)

-What about premature vascular disease?

- looked at those with age of onset ≤ 60 or ≤ 55 but absolutely no difference (Table 4)

Blood pressure

- did notice in original analysis that a greater % of non-carriers were diagnosed with HT/on anti-HT meds (66/393 non carriers (16.8%) vs. 7/118 carriers (8.5%) $p=0.026$)
(this includes individuals with no pre-medication lipid levels)
- Why wasn't blood pressure different between the groups in table 1?
 - individuals taking other anti-HT meds were included, and these were treated bp's
- When look at pre-treatment bp's see significant differences between carriers and non-carriers in several age groups, and in σ and φ (see packet of tables from Jennifer and Odell)

Best stats for lipids: excludes those on all meds which might affect lipids (lipid lowering, diuretics, b-blockers, HRT, IGT, steroids, testosterone, anti-epileptics); those with diabetes, IGT; those who were E2/2, pregnant, had kidney or liver disease, LPL207 or 188 mutations (in French Canadians) and carriers of the D9N and N291S mutations (EXCLALL)

Age \geq 18, males and females

Stats: 2 tailed T-test, $p < 0.05$ sig.

Table 1. Baseline demographics

	carriers			noncarriers			p value
	n	mean	SD	n	mean	SD	
age	91	41.5	14	316	43	13.4	0.39
BMI	82	24.39	3.64	291	24.69	3.82	0.52
m/f		45/46			141/175		0.47
glucose	86	5.12	0.41	302	5.17	0.42	0.42
sys bp	82	128.0	15.6	293	127.6	15.8	0.86
dias bp	82	80.4	9.5	293	81.4	10.6	0.41

Table 2. Lipid values in carriers vs. non-carriers

TG	91	1.21	0.47	306	1.52	0.67	<0.001
logTG	91	0.051	0.173	306	0.141	0.19	<0.001
TC	91	8.99	1.89	316	8.86	1.62	0.90
HDL	90	1.26	0.32	309	1.26	0.34	0.90
LDL	90	7.06	1.86	305	6.84	1.56	0.30
TC/HDL	90	7.49	2.77	309	7.50	2.64	0.97
TG/HDL	90	1.02	0.47	304	1.34	0.82	<0.001
Vasc. dis.	12/91	13.20%		53/316	16.80%		0.52
age vascular disease onset	12	44.4	12.7	53	48.5	12.0	0.33

similar results if males and females examined separately

Vascular disease: CHD, PVD and/or CVA

diagnosis: MI, CABG, PTCA, angina treated with medication, CHD on angiogram, stroke, TIA

claudication and surgery on carotid or abdominal arteries treated due to atherosclerosis

Does not include: bruits only, aneurysms, ultrasound results only or any other ambiguous diagnosis

Stats: Fishers exact test

Better stats: put back in people who were lacking premed lipids or were on other lipid affecting drugs
diabetics still excluded (IGT included)- EXCL13**Table 3. Prevalence of vascular disease**

	carriers	%	non carriers	%	p
vascular disease	19/117	16.2	88/404	21.8	0.24
age onset	49.0±11.4		50.1±12.2		0.33
Males:					
vasc. dis.	12/56	21.4	53/174	30.5	0.30
Females:					
vasc. dis.	7/63	11.1	35/230	15.2	0.54

OR: like before but only put people back in who we had no premed lipids on (still exclude other
meds affecting lipids, diabetes, IGT etc.)- EXCLSC2

	carriers	%	non carriers	%	p
vascular disease	13/93	14	62/355	17.5	0.36
males	9/46	19.6	38/150	25.3	0.55
females	4/47	8.5	24/185	13	0.62

subdividing by males and females makes numbers too small

} best in terms
of criteria.

?Premature vascular disease: look at above groups but only those with age onset "premature"

Table 4. Prevalence of Premature vascular disease

ageonset<=60	carriers	%	non carriers	%	p
excl11	11/91	12.1	43/316	13.6	0.86
excl13	17/117	14.5	68/404	16.8	0.67
exclsc2	12/93	12.9	51/355	14.4	0.87
ageonset<=55	carriers	%	non carriers	%	p
excl11	11/91	12.1	38/316	12	1
excl13	16/117	13.7	58/404	14.4	1
exclsc2	12/93	12.9	45/355	12.7	1

is there an age? age of onset in all (19) carriers with vasc. dis: 42,48,26,26,55,47,46,32,72,42,47,
50,42,53,46,53,49,62,59

ANALYSIS OF HYPERTENSION AND BP LOWERING MEDS

TOTAL: 650
 - excl = 567
 - DM + IGT = 511

	TOTAL	Carrier +/-	non carrier -/-	P value
	511	118	393	
▪ ON BP LOWERING MEDS:	64	10 (8.5%)	54 (13.7%)	p=0.13
diuretics + beta blockers: for HT:	6 (4)	2 (1)	4 (3)	
diuretics alone or in combination: for HT:	20 (16)	3 (2)	17 (14)	
beta blockers alone or in combination: for HT:	14 (6)	1 (1)	13 (5)	
ACE inhibitors alone: for HT:	8 (8)	0	8 (8)	
Ca channel blockers alone: for HT:	14 (7)	4 (3)	10 (4)	
ACE inhibitors + Ca channel blockers: for HT:	2 (2)	0	2 (2)	
▪ ON BP LOWERING MEDS for HT:	43	7 (5.9%)	36 (9.2%)	p=0.27
▪ ON BP LOWERING MEDS NOT for HT:	21	3	18	
• Patients diagnosed with HT, but NOT ON BP MEDS:	33	3	30	
> Current HT, NO meds at visit:	16	1	15	
> Hx of HT, but not current:	17	2	15	
▪ TOTAL WITH HT, (on and off BP meds):	76	10 (8.5%)	66 (16.8%)	p=0.026

BREAKDOWN OF PATIENTS WITH HYPERTENSION

Of the 93 patients total with essential hypertension:

- 75 were diagnosed as having HT at the time of their visit
- 18 were described as having a past history of HT but were not currently suffering from HT.

Of these 75 currently with hypertension:

- 8 were being treated with a combination of beta blockers and diuretics
- 18 were taking diuretics alone
- 13 were on beta blockers alone
- 2 were prescribed beta blockers to treat their angina and were taking other medications for their HT
- 25 were on other anti-hypertension medications
(captopril, diltiazem, enalapril, lisinopril, nifedipine, norvasc, prazosin, triamterene)
- 9 were apparently receiving no treatment at the time of their visit
-5 of which were described as having HT in their consultation letters
-while 4 individuals were diagnosed with essential HT by John

Table 1
Whole group (related): Off Medication Blood Pressure - diastolic and systolic
Comparison between different age groups
excl 5 column: including patients with and without pre-medication lipids, excluding patients with DM or IGT

Age Groups (years)	Blood Pressure Diastolic				Blood Pressure Systolic			
	Set447Ter Carriers n	mean	stdev	P-value	Set447Ter Carriers n	mean	stdev	P-value
ALL	101	77.79	10.32	0.000-	101	124.96	17.83	0.01
<= 20	10	63.8	8.3	0.004	10	101.6	17.2	0.039
<= 25	23	68.52	10.14	0.014	23	110	17.8	0.035
<= 30	34	71.59	10.8	0.028	34	113	17.16	0.081
<= 40	54	74.52	10.31	0.013	54	117.02	15.48	0.039
<= 50	67	76.24	10.52	0.003	67	119.09	15.32	0.005
<= 60	89	77.71	10.44	0.001	89	123.37	17.57	0.024
20 <=> 70	90	79.41	9.36	0.002	90	127.43	16.08	0.058
20 <=> 60	79	79.47	9.33	0.012	79	126.13	15.68	0.113
20 <=> 50	57	76.42	9.32	0.03	57	122.16	12.85	0.029
20 <=> 40	44	76.96	9.15	0.11	44	120.52	12.87	0.176
>= 20	91	79.33	9.34	0.001	91	127.53	16.02	0.05
>= 30	70	80.9	8.66	0.018	70	130.56	15.09	0.298
>= 40	49	81.29	9.02	0.006	49	133.74	15.8	0.305
>= 50	34	80.85	9.32	0.013	34	136.53	16.92	0.501
>= 60	13	78.54	9.37	0.021	13	137.38	15.29	0.381

Whole group (related): Off Medication Blood Pressure - diastolic and systolic
Comparison between genders within different age groups
excl 5 column: including patients with and without pre-medication lipids, excluding patients with DM or IGT

Age Groups (years)	Blood Pressure Diastolic				P-value	Blood Pressure Systolic				P-value				
	Serial 477er Carriers n	mean	sidev	Non-carriers n		mean	sidev	Serial 477er Carriers n	mean		sidev	Non-carriers n	mean	sidev
ALL AGES	101	77.79	10.32	360	82.46	11.19	0.000-	101	124.96	17.83	360	130.23	18.92	0.01
males	44	80.432	9.33	164	83.87	10.75	0.002	44	126.77	14.41	164	130.6	17.72	0.06
females	57	75.75	10.66	196	81.44	11.48	0.001	57	124.39	20.19	196	129.92	19.9	0.071
>=20	91	79.33	9.34	336	83.12	11.1	0.001	91	127.53	16.02	336	131.38	18.14	0.05
males	41	81.49	8.69	149	84.68	10.55	0.002	41	127.34	14.7	149	132.18	16.04	0.045
females	50	77.56	9.57	187	81.89	11.4	0.008	50	127.68	18.42	187	130.75	19.66	0.305
>=30	70	80.9	8.66	294	83.83	11.08	0.018	70	130.58	15.09	294	132.75	18.17	0.298
males	33	83.21	7.38	128	85.78	10.77	0.101	33	129.36	10.69	128	133.11	16.15	0.015
females	37	78.84	9.27	166	82.71	11.5	0.032	37	131.62	18.23	166	132.46	19.63	0.803
>=40	49	81.29	9.02	213	85.46	10.97	0.006	49	133.74	15.8	213	136.39	18.68	0.305
males	22	82.64	7.99	95	85.9	10.65	0.115	22	130.36	11.54	95	134.92	16.96	0.138
females	27	80.16	9.76	118	85.12	11.25	0.027	27	136.44	18.33	118	137.58	19.94	0.776
>=50	34	80.85	9.32	132	85.62	10.69	0.013	34	136.53	16.92	132	138.8	19.1	0.501
males	11	82.18	5.9	53	85.32	9.92	0.002	11	133.46	10.96	53	134.92	18.12	0.726
females	23	80.22	10.64	79	85.82	11.24	0.034	23	138	19.17	79	141.39	19.41	0.461
>=60	13	78.54	9.37	56	85.88	10.04	0.021	13	137.38	15.29	56	141.64	15.84	0.381
males	1	88		22	86.04		insuff. data		132		22	138.91		insuff. data
females	12	77.75	9.32	34	86.76	11.3	0.024	12	137.83	15.88	34	143.41	15.77	0.308

Table 3

Whole group (related): Off Medication Blood Pressure - diastolic and systolic
 Comparison between genders within different age groups
 excel 5 column: including patients with and without pre-medication lipids, excluding patients with DM or IGT

Age Groups (years)	Blood Pressure Diastolic				Blood Pressure Systolic			
	Set447Ter Carriers n	mean	sidev	P-value	Set447Ter Carriers n	mean	sidev	P-value
20 <=> 40	44	76.96	9.15	0.111	44	120.52	12.87	0.176
	males 20	79.65	9.49		males 20	123.64	13.85	
20 <=> 40	females 24	74.71	8.4	0.267	females 24	120.2	13.3	0.445
	males 20	79.65	9.49		males 20	123.64	13.85	
20 <=> 50	57	78.42	9.32	0.03	57	122.16	12.85	0.029
	males 30	81.23	9.58		males 30	130.85	14.58	
20 <=> 50	females 27	75.3	8.08	0.039	females 27	123.06	15.83	0.145
	males 30	81.23	9.58		males 30	130.85	14.58	
20 <=> 60	79	79.47	9.33	0.012	79	126.13	15.68	0.113
	males 40	81.32	8.73		males 40	130.88	15.55	
20 <=> 60	females 39	77.56	9.65	0.05	females 39	128.13	19.2	0.346
	males 40	81.32	8.73		males 40	130.88	15.55	
20 <=> 70	90	79.41	9.36	0.002	90	127.43	16.08	0.058
	males 41	81.49	8.69		males 41	132.18	16.04	
20 <=> 70	females 49	77.67	9.64	0.011	females 49	130.37	19.59	0.347
	males 41	81.49	8.69		males 41	132.18	16.04	

Table 4

Whole group (related): OR Medication Blood Pressure - diastolic and systolic
 Comparison between genders within different age groups
 excel 5 column: including patients with and without pre-medication lipids, excluding patients with DM or IGT

Age Groups (years)	Blood Pressure Diastolic				Blood Pressure Systolic			
	Ser447Ter Carriers		Non-carriers		Ser447Ter Carriers		Non-carriers	
	n	mean	stdev	P-value	n	mean	stdev	P-value
<= 20	10	63.8	8.3	0.004	10	101.6	17.2	0.039
	males 3	66	5.29	0.009	3	103.33	20.82	0.334
	females 7	62.86	9.51	0.009	7	100.86	17.24	0.161
<= 25	23	69.52	10.14	0.014	23	110	17.8	0.035
	males 9	70.33	8.86	0.075	9	108.44	18.7	0.24
	females 14	69	11.17	0.007	14	108.43	17.73	0.111
<= 30	34	71.58	10.6	0.028	34	113	17.16	0.0817
	males 12	73.58	10.77	0.215	12	108.93	19.28	0.439
	females 22	70.5	10.59	0.007	22	110.91	15.96	0.201
<= 40	54	74.52	10.31	0.013	54	117.02	15.48	0.039
	males 23	77.87	10.12	0.007	23	121.7	16.35	0.259
	females 31	72.03	9.88	0.034	31	114.06	15.16	0.094
<= 50	67	76.24	10.52	0.003	67	119.09	15.32	0.005
	males 33	79.85	10.24	0.139	33	123.12	14.63	0.076
	females 34	72.74	9.7	0.003	34	115.18	15.17	0.021
<= 60	89	77.71	10.44	0.001	89	123.37	17.57	0.024
	males 43	80.26	9.37	0.005	43	125.56	14.55	0.165
	females 46	75.33	10.92	0.005	46	121.33	19.93	0.075

24-06-1999

EP99202048.7

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CLAIM

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Use of the LPL S447X mutant gene for the preparation of a gene therapeutic composition for the treatment of angina pectoris, for protection against cardiovascular disease and for lowering elevated blood pressure.